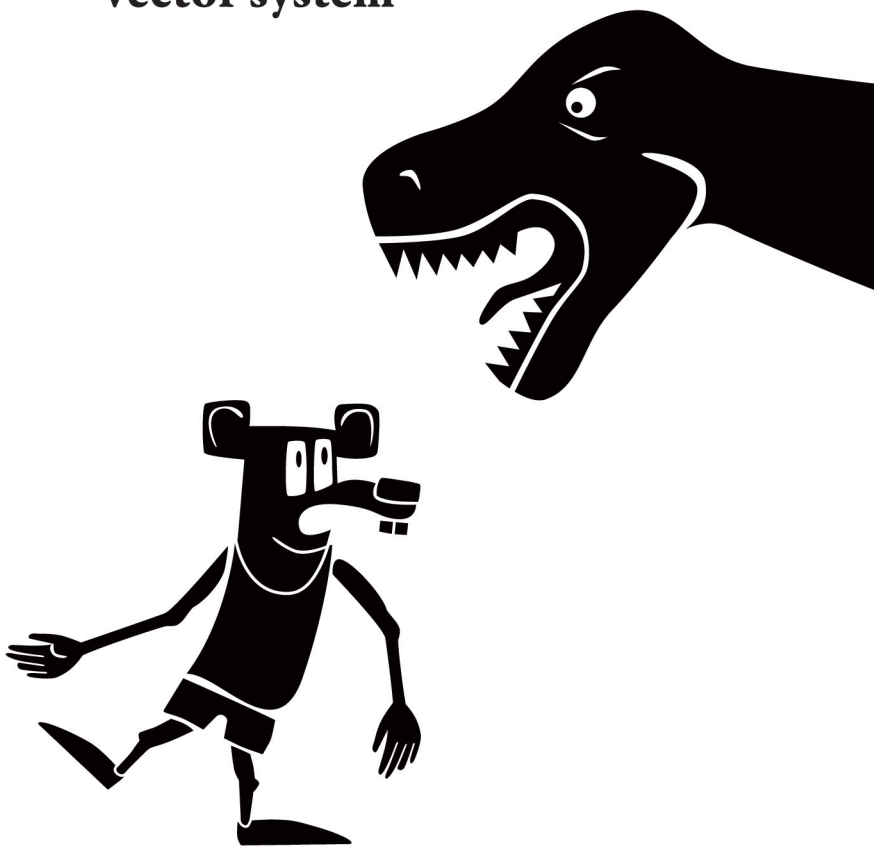
Alphavirus-based therapeutic immunization against cervical neoplasia

Walczak, Mateusz

CHAPTER 5

Role of regulatory T-cells in immunization strategies involving a recombinant alphavirus vector system



Mateusz Walczak, Joke Regts, Antoon J.M. van Oosterhout, Louis Boon, Jan Wilschut, Hans W. Nijman, Toos Daemen

Abstract

Background: Regulatory T cells (Treg) hamper immune responses elicited by cancer vaccines. Therefore, depletion of Treg is being used to improve the outcome of vaccinations.

Methods: We studied whether an alphavirus vector-based immunotherapeutic vaccine changes the number and/or activity of regulatory T cells (Treg) and if Treg depletion improves the efficacy of this vaccine against tumors. The vaccine is based on a Semliki Forest virus (SFV). The recombinant SFV replicon particles encode a fusion protein of E6 and E7 from HPV16 (SFVeE6,7).

Results: We demonstrated that SFVeE6,7 immunization did not change Treg levels and their suppressive activity. Depletion of Treg in mice, using the novel anti-FR4 (Folate Receptor 4) antibody, did not enhance the immune response induced by SFVeE6,7 immunization. Both, the priming and the proliferation phase of the HPV-specific response elicited with SFVeE6,7 were not affected by the immune-suppressive activity of Treg. Moreover, Treg depletion did not improve the therapeutic antitumor response of SFVeE6,7 in a murine tumor model.

Conclusions: The efficacy of the SFVeE6,7 vaccine is not hampered by Treg. Therefore SFVeE6,7 seems a very promising candidate for the treatment of HPV-induced disease, as it may not require additional immune interventions to modulate Treg activity.

Introduction

Cancer vaccine-induced immune responses have been described to be hampered by immunosuppressive regulatory T cells (Treg).[1-8] Immunization itself may result in an increased frequency of Treg.[9-14] Next, cancer patients frequently already have increased numbers of Treg .[15-18] In a previous study we reported on the increased number and suppressive activity of Treg in patients with cervical intraepithelial neoplasia (CIN) and cervical cancer.[16] Inhibition of these immune suppressors along with immunization protocols might therefore augment vaccine-induced responses. However, such strategies need careful consideration as Treg are also essential to maintain immune homeostasis and are involved in preventing autoimmunity.[19-21]

Immunizations with recombinant vaccinia virus, dendritic cells, adenylate cyclase, peptide, DNA and Listeria-based vaccines have been described to result in increased numbers of Treg.[9-14,22,23] Vaccination with recombinant vaccinia virus, encoding hemaglutinin from influenza virus, expanded the number of Treg in spleen as much as 30-fold.[14] To overcome this phenomenon and to enhance immune responses, the effect of temporal depletion or inactivation of Treg in combination with immunotherapeutic strategies is currently being evaluated in mice and humans.[1-8]

We have developed a viral vector-based therapeutic vaccine against malignancies induced by human papillomavirus (HPV). The recombinant viral vector, which is based on an alphavirus, Semliki Forest virus (SFV), encodes a fusion protein of E6 and E7 from HPV type 16 (SFVeE6,7).[24,25] We demonstrated that immunization with SFVeE6,7 replicon particles results in strong HPV-specific cellular responses and eradication of established HPV-transformed tumors. Moreover tolerance can be broken in transgenic mice immune tolerant for HPV.[26-29] The present study was set out to explore, whether SFV-based immunization strategies can be further optimized by regimes to suppress Treg.

One of the strategies to suppress Treg is to use depleting antibodies specific for Treg. Treg are a phenotypically heterogeneous population co-expressing markers such as CD4, CD25,

CTLA4, GITR and Foxp3.[30,31] The transcription factor Foxp3 is one of the key controllers of Treg development and its expression is essential to establish a functional regulatory T cell lineage.[32-36]

Although CD25 is also expressed on activated T cells, anti-CD25 antibodies are often used to deplete Treg in animal model studies and recently also in clinical studies.[2,4,37-39] However, depletion of Foxp3$^+$ Treg using anti-CD25 is partial and short-lived.[40,41] Furthermore, recently Curtin *et al.* showed that Treg depletion in mice using PC61 (one of the anti-CD25 clones) also inhibits clonal expansion of tumor antigen-specific T cells.[42]

A very promising novel antibody to study the role of Treg depletion on immune responses is an antibody recognizing folate receptor 4 (FR4). Treg constitutively express high amounts of FR4 distinguishing them from other naive or activated T cells. Administration of anti-FR4 antibody as such, i.e. without concomitant immunization, already induces anti-tumor immunity in tumor-bearing mice.[43]

In this study we first determined if SFVeE6,7 immunization, as described for other cancer immunization strategies, results in an enhanced number or activity of Treg. Next we evaluated if combining SFVeE6,7 prime-boost immunization with Treg depletion through co-administration of anti-FR4 antibody further improves the immune effectiveness of the SFVeE6,7 immunization strategy.

Material and methods

Cell lines

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (# CCL-10). C3 cells, 13-2 cells and TC-1 cells were a kind gift from Prof. C. Melief and Dr. R. Offringa (Leiden University Medical Center, The Netherlands). All cells were cultured as described previously.[27]

Mice

Specified pathogen-free female C57BL/6 mice were used at 6 to 10 weeks of age. They were purchased from Harlan CPB (Zeist, The Netherlands) and kept according to institute guidelines. All animal experiments were approved by the local Animal Experimentation Ethical Committee.

Production, purification and titer determination of SFVeE6,7 particles

The production, purification and titer determination of recombinant SFV-eE6,7 was performed as described previously.[24] In brief, pSFV3-eE6,7 was produced using pSFV-Helper 2 and purified on a discontinuous sucrose density gradient. Before use, SFV particles were activated with 1/20 volume of α-chymotrypsin (10mg/ml; Sigma Chemical Co., St. Louis, MO, USA). SFV particles were titrated using BHK-21 cells. pSFV3-eE6,7 encodes an enhanced

expression of a fusion product of E6 and E7 of HPV type 16.

SFVeE6,7 immunizations and anti-FR4 treatment

Mice were immunized i.m. with different amounts of SFVeE6,7 (10^5-10^7 particles), followed by one booster immunization with a two-week interval. One day before each immunization, in some experiments, mice were injected i.p. with 25 μg anti-FR4 (TH6 clone) antibody. Anti-FR4 antibody was a kind gift from Prof. S. Sakaguchi (Kyoto University, Japan). Mice injected i.m and/or i.p. with PBS were negative controls.

Treg, MHC class I tetramer staining and FACS analysis

Spleen cells and PBMCs obtained from immunized and control mice at the indicated time points were stained with FITC-conjugated anti-CD8 (ProImmune, Oxford, UK) and PE-conjugated MHC class-I tetramers loaded with RAHYNIVTF (HPV16 E7 epitope) (Sanquin, Amsterdam, The Netherlands). It allowed us to determine the frequency of HPV-specific CTLs. To analyze Treg numbers, cells were stained with PE-conjugated anti-CD4 (SouthernBiotech, Birmingham, AL, USA), APC-conjugated anti-CD25 (eBioscience, San Diego, CA, USA), FITC-conjugated anti-CD127 (eBioscience, San Diego, CA, USA). After washing, cells were permeabilized with Foxp3 staining buffer set according to the manufacturer's instructions (eBioscience, San Diego, CA, USA) and additionally stained with Alexa Fluor® 700-conjugated anti-Foxp3 (eBioscience, San Diego, CA, USA). Cells were washed and measured by flow cytometry (LSR-II from BD Biosciences, Erembodegem, Belgium). Collected data was analyzed with the Win-List software package (Verity Software House Inc., ME, USA).

Cell sorting

Freshly isolated splenocytes from SFVeE6,7 or PBS immunized mice were stained with PE-conjugated anti-CD4 (SouthernBiotech, Birmingham, AL, USA) and APC-conjugated anti-CD25 or FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 (all from eBioscience, San Diego, CA, USA). Cells were washed and sorted on a FACS MoFlo (Becton & Dickinson) according to their forward and side scatter properties. Cells were sorted into $CD4^+CD25^{med/high}$ (Treg) and cells depleted from Treg. The purity of the sorted Treg and depleted cells, as determined by flow-cytometric reanalysis, was >85% and >90%, respectively (Figure 2A and 3A).

Regular CTL and micro-CTL assay

In regular CTL assay, 10 days after the last immunization, spleen cells were isolated and co-cultured with irradiated (100 Gy) TC-1 cells in a ratio of 25:1, in 25 cm^2 culture flasks placed upright. In some experiments, before restimulation, spleen cells were depleted from Treg by cell sorting. After a 5- or 7-day *in vitro* restimulation culture, cells were harvested and CTL

activity was determined in a standard 4 h ^{51}Cr release assay. In micro-CTL assay spleen cells were co-cultured with TC-1 cells in 96-wells plates. To some cultures freshly sorted Treg were added in the different ratios. After 5- or 7-day *in vitro* restimulation, cells from micro-CTL assays were not harvested, but target cells were directly added to these cultures. Target cells (C3 cells) were labeled for 1 h with 3.7 MBq ^{51}Cr/10^6 cells in 100 µl medium (MP Biomedicals, Inc., Irvine, CA, USA). The mean percentage of specific ^{51}Cr-release was calculated according to the formula: % specific release = [(experimental release − spontaneous release) / (maximal release − spontaneous release)] cpm × 100. The spontaneous ^{51}Cr-release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the mean.

Proliferation assay

Freshly isolated spleen cells ($1.5x10^5$) were cultured alone or co-cultured with different amounts of freshly sorted Treg in round-bottomed 96-well plates in 150 µl medium. Cells were incubated in the presence or absence of soluble anti-CD3 (1 µg/ml; clone 145-2C11) and soluble anti-CD28 (1 µg/ml; clone PV-1) (both provided by Bioceros, Utrecht, The Netherlands) for 4 days. For the last 18 hours of culture, 75 µl of the supernatant was removed, and 0.5 µCi/well [^{3}H]-thymidine (ICN Biomedical Inc., Costa Mesa, CA, USA) was added. Cells were then harvested onto 96-wells filters using a Mach III M Harvester (Tomtec, Inc. Hamden, CT, USA) and sealed in plastic bags containing 6 ml Betaplate Scint cocktail (Wallac Inc., Turku, Finland). [^{3}H]-thymidine incorporation was counted on a 1450 Microbeta Trilux β-scintilation counter (Wallac Inc., Turku, Finland) using Wallac 1450 Microbeta Trilux & Microbeta Jet software. Results are expressed as counts per minute (cpm).

Tumor treatment experiments

Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells suspended in 0.2 ml Hank's Balanced Salt Solution (HBSS, Invitrogen, Paisley, UK). Ten days later the mice were immunized i.m. with 10^6 (low dose) or $5x10^6$ (high dose) SFVeE6,7 particles and boosted twice with a one-week interval. For Treg depletion the mice were injected i.p. with 25 µg anti-FR4 one day before each SFVeE6,7 immunization. Control mice were injected i.m. and/or i.p. with PBS. The same skilled technician performed the tumor measurements. When the tumor grew through the skin or if the tumor volume exceeded 1000 mm^3, the mice were killed.

Statistical analysis

Data are presented as the mean ± SD. Data were analyzed using Student t-Test. The Cox proportional hazards test was used for statistical analysis of tumor treatment responses. Statistical significance was defined as $P < 0.05$ (or as indicated).

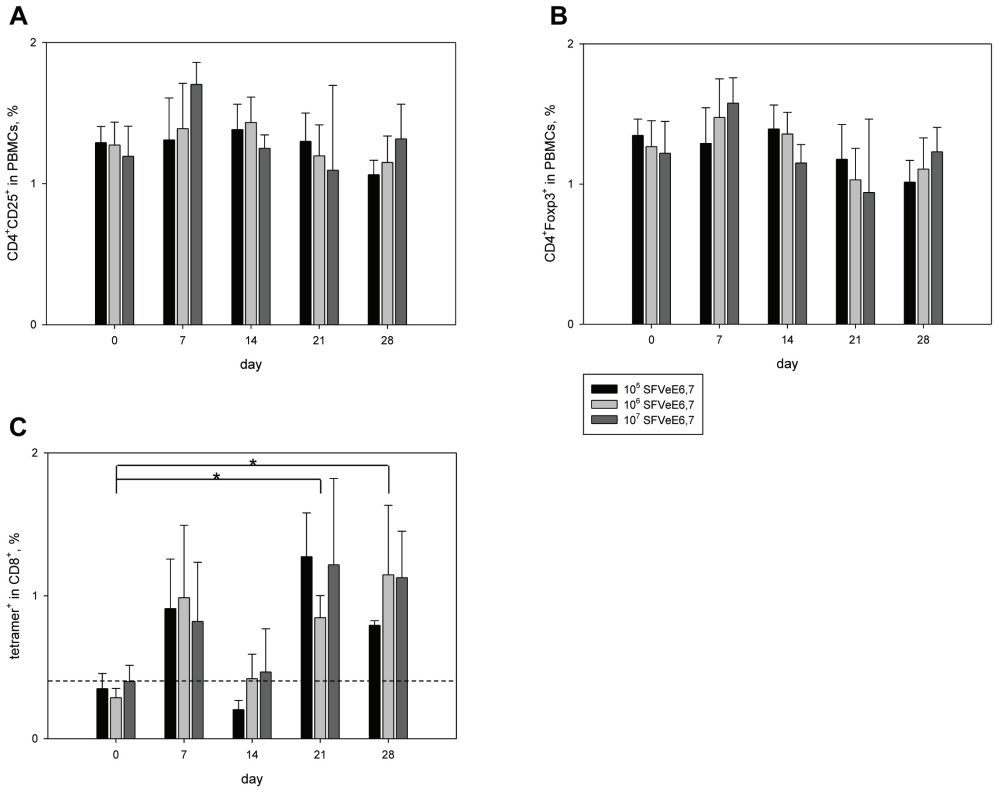


Figure 1. Immunization with SFVeE6,7 did not increase Treg levels

Mice were immunized and boosted i.m. with 10^5(n=3) 10^6 (n=3) and 10^7 (n=3) SFVeE6,7 on day 0 and 14. Starting from day 0, every 7 days blood from each animal was taken and the levels of (A) $CD4^+CD25^+$ and (B) $CD4^+Foxp3^+$ cells were determined. Moreover, frequencies of (C) HPV-specific $CD8^+$ cells were determined. The dashed line represents background level. Error bars represent standard deviation. *P < 0.05.

Results

Induction of suppressor and effector T cells by SFVeE6,7 immunization

Intravenous (i.v.) or intramuscular (i.m.) immunization with SFVeE6,7 induces strong cellular and anti-tumor responses in mice.[24,26-29] We reasoned that this strong immune activation might also induce a suppressive immune response, as described for other immunization strategies.[9-14,22,23,44] To investigate the influence of SFVeE6,7 immunization on induction of Treg, we analyzed the frequencies of Treg and HPV-specific cytotoxic T lymphocytes (CTL) in blood and spleen of mice immunized with SFVeE6,7. Treg were analyzed by flow cytometry based on the expression of $CD4^+CD25^+$ and $CD4^+Foxp3^+$. HPV-specific $CD8^+$ T cells were stained using $E7_{49-57}$ MHC class I tetramers.

PBMCs were analyzed weekly throughout the immunization period and spleens were analyzed ten days after the booster immunization. At all time points analyzed, prime-boost

immunizations up to 10^7 infectious units (i.u.) SFVeE6,7 did not change the frequency of $CD4^+CD25^+$ T cells (Figure 1A) or $CD4^+Foxp3^+$ T cells (Figure 1B) in blood. Also in spleen, no significant changes of these populations were observed ten days after the booster immunization (data not shown). The frequency of HPV-specific T cells within the $CD8^+$ T cell population, with all dosages of SFVeE6,7 tested, increased to approximately 1% after the prime immunization. This frequency declined on day 14 and significantly increased again after the booster immunization (Figure 1C; $P < 0.05$). These results suggest that SFVeE6,7 immunization does not augment the number of Treg in mice, but boosts HPV-specific CTL.

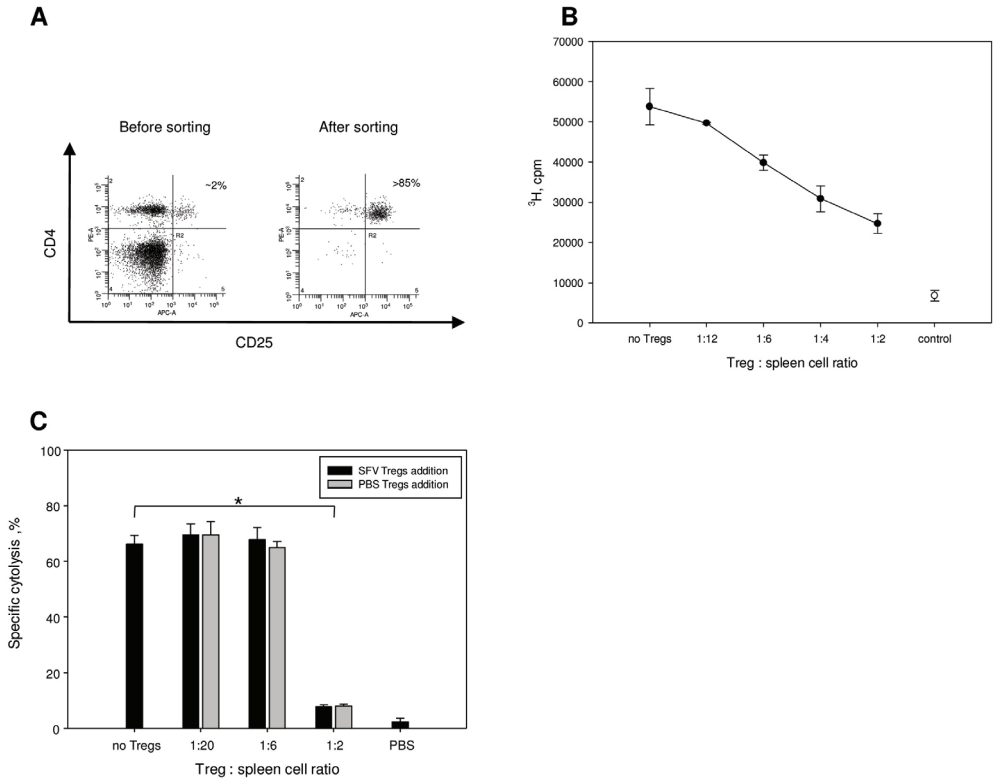


Figure 2. The influence of Treg addition on proliferation and lytic activity of HPV-specific CTL *in vitro*

Mice were immunized with 10^6 SFVeE6,7 or PBS on day 0 and 14. Ten days after the boost the mice were killed and spleen cells isolated. Treg were obtained from SFVeE6,7 and PBS treated mice by cell sorting based on $CD4^+CD25^{med/hi}$ expression. (A) A representative example of sorting purity is shown. (B) The sorted $CD4^+CD25^{med/hi}$ Treg were added to spleen cells (isolated from naive mice) in different ratios. After 4 days *in vitro* restimulation, in the presence of anti-CD3 and anti-CD28, cell proliferation was measured by ^{3}H-thymidine uptake. The cpm as determined in triplicate well assay are shown, with error bars representing standard deviation. (C) Spleen cells, isolated from 10^6 SFVeE6,7 immunized mice were co-cultured with different numbers of freshly sorted Treg (from 10^6 SFVeE6,7 or PBS injected mice). After 7 days *in vitro* restimulation ^{51}Cr release was performed. The percentages of specific cytolysis at different spleen cells to Treg ratios are shown. Error bars represent standard deviation. *$P < 0.002$.

Influence of Treg addition on proliferation and lytic activity of HPV-specific CTL *in vitro*

Despite the fact that no Treg were induced by SFVeE6,7, existing Treg may affect the proliferation and/or lytic activity of antigen-specific T cells induced by SFVeE6,7 immunization. We therefore investigated the effect of Treg on *in vitro* specific-CTL responses induced by SFVeE6,7. Treg were isolated from mice treated with SFVeE6,7 and from control mice by positive sorting for CD4 and CD25 cell surface expression. Purity of samples after sorting was above 85% (Figure 2A). Approximately 90% of the sorted $CD4^+CD25^{med/hi}$ cells expressed Foxp3 (data not shown). The isolated Treg suppressed proliferation of anti-CD3 and anti-CD28 stimulated spleen cells (Figure 2B). In the spleens of naïve mice 1 in 50 spleen cells is a regulatory T cell.

The sorted $CD4^+CD25^{med/hi}$ Treg were added to *in vitro* restimulation cultures of spleen cells isolated from SFVeE6,7-immunized mice. As the number of Treg that can be isolated from a mouse spleen is low, we set up a micro CTL assay. In this assay the spleen cells are cultured in 96-well plates and after the 7-day restimulation with irradiated HPV-specific tumor cells, ^{51}Cr-labeled target cells are added to the restimulation cultures.

The cytolytic activity of the CTL was not influenced by the addition of Treg (Figure 2C). As SFV immunization does not result in increased numbers of Treg also in the spleen of immunized mice there is 1 Treg per 50 splenocytes. As 10% of the spleen cells is $CD8^+$ and 1% of the $CD8^+$ is E7-specific, this implies that in these mice there is 100 Treg per 5 E7-specific T cells. Only the addition of 25-fold more Treg (i.e. 1000 Treg per 2 E7-specific $CD8^+$ T cell; in the Figure 2C represented by ratio 1 Treg : 2 spleen cells ratio) resulted in a decrease in CTL activity. This decrease is due to suppression of proliferation of HPV-specific $CD8^+$ T cells during the restimulation protocol as in a separate experiment we demonstrated that addition of Treg in the 4hr ^{51}Cr-release assay did not influence cytolysis (not shown). It should be noted that no differences were observed between the activity of Treg isolated from naïve or SFVeE6,7-immunized mice (Figure 2C). Thus, Treg isolated from naïve or SFVeE6,7-immunized mice, up to a ratio of 1 Treg to 6 spleen cells, do not change the *in vitro* proliferation and lytic activity of HPV-specific CTL elicited by SFVeE6,7 immunization.

Changes in HPV-specific CTL responses after *in vitro* depletion of Treg

We next examined if depletion of $CD4^+CD25^{med/hi}$ T cells, of which more than 90% were $Foxp3^+$, enhances the *in vitro* proliferation and/or activity of HPV-specific T cells. Spleen cells isolated from SFVeE6,7-immunized mice were depleted from $CD4^+CD25^{med/hi}$ cells by cell sorting and subsequently restimulated *in vitro* before being tested in a ^{51}Cr-release assay. As a control, $CD4^+CD25^{med/hi}$-depleted spleen cells were also reconstituted with $CD4^+CD25^{med/hi}$ T cells to the original ratio found in the spleen. In addition, *in vitro* restimulated cultures of unsorted spleen cells from SFVeE6,7-immunized mice and spleen cells from non-immunized

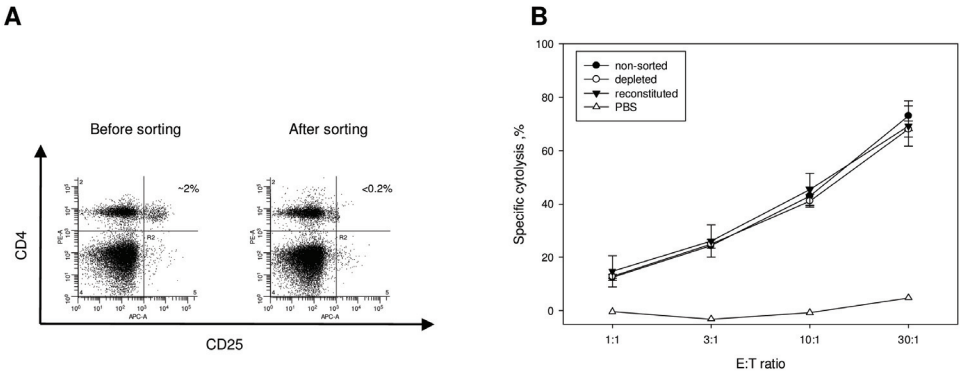


Figure 3. Depletion of Treg before *in vitro* restimulation culture did not increase CTL responses

On day 0 and 14 mice (n=3) were immunized with 10^6 SFVeE6,7 or injected with PBS (n=1). Ten days after the last injection mice were killed and spleen cells isolated. Before 7 days *in vitro* restimulation, Treg were depleted using cell sorting. (A) A representative example of sorting purity is shown. (B) Cytolytic activity of sorted, non-sorted and reconstituted spleen cells (sorted and than pooled) were analyzed in a CTL assay. The percentages of specific cytolysis, measured in triplicate assay, against target cells at different E:T ratios are shown. Error bars represent standard deviation.

control mice were analyzed. In Figure 3A a typical flow cytometry analysis of one of the spleen populations before and after sorting is depicted. The sorted population contained less than 0.2% $CD4^+CD25^{med/hi}$ T cells as opposed to 2% in the unsorted population. Depletion of $CD4^+CD25^{med/hi}$ T cells before *in vitro* restimulation did not influence the cytolytic activity of HPV-specific CTL compared to the activity measured in reconstituted and unsorted spleen cells (Figure 3B). Thus, depletion of Treg does not enhance SFVeE6,7-specific CTL response.

Influence of *in vivo* Treg depletion on the immune response induced by SFVeE6,7 immunization

Growing evidence in literature indicates that immune responses induced by immunization are enhanced after *in vivo* Treg elimination.[1-8] We therefore set out to investigate if *in vivo* depletion of Treg can also enhance the *in vivo* induction of HPV-specific CTL by SFVeE6,7 immunization.

As still relatively few studies have been performed with anti-FR4, the efficacy of this antibody to deplete Treg *in vivo* was investigated. Mice were injected with a single dose of anti-FR4. At the indicated time points, PBMCs were analyzed by flow cytometry. As presented in Figure 4A and B, injection of 25 μg anti-FR4 strongly suppressed the level of $CD4^+Foxp3^+$ cells in PBMCs. One and fourteen days after antibody injection, the frequency of $CD4^+Foxp3^+$ T cells was approximately 20% and 60% of the control value, respectively ($P < 0.005$). Anti-FR4 injection depleted specifically $Foxp3^+CD25^+$ cells, leaving $Foxp3^-CD25^+$ population unaffected (Figure 4C; $P < 0.005$). Moreover anti-FR4 administration did not change the levels of $CD8^+$ T cells. The decrease in $CD4^+$ T cells can be mainly attributed to loss of $CD4^+FoxP3^+$ cells (Figure 4D; $P < 0.005$).

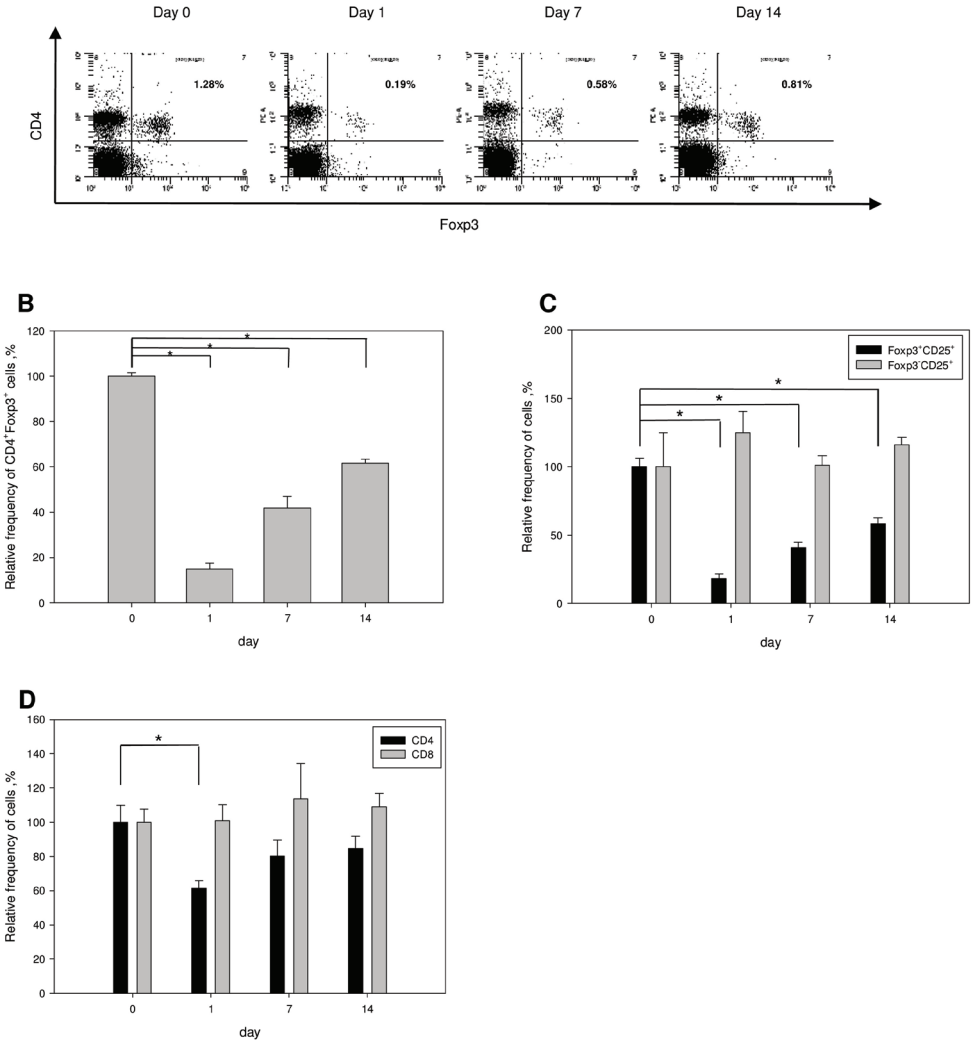


Figure 4. Anti-FR4 antibody strongly decreased Treg frequencies in blood

Mice (n=3) were injected i.p. with 25 μg anti-FR4 on day 0. At the indicated time points PBMCs were stained with CD4, CD8, CD25 and Foxp3. (A) A representative FACS analysis of $CD4^+Foxp3^+$ cells is shown. (B-D) The changes in percentage of different cell subsets, after anti-FR4 injection, with PBS control set as 100% are shown. Error bars represent standard deviation *P < 0.005.

Next, we combined anti-FR4 antibody treatment with SFVeE6,7 immunization. Mice were immunized twice (on day 0 and 14) with 10^6 i.u. SFVeE6,7 and one day prior to each immunization anti-FR4 was administered. Under these conditions, approximately 80% of the naturally occurring $CD4^+Foxp3^+$ T cells was depleted at the time of SFVeE6,7 immunization (Figure 4B). However despite this strong Treg depletion, the frequency of SFVeE6,7-induced HPV-specific $CD8^+$ T cells in spleen, as determined 10 days after the booster immunization, was not higher than the frequency reached after SFVeE6,7 immunization alone (Figure 5A).

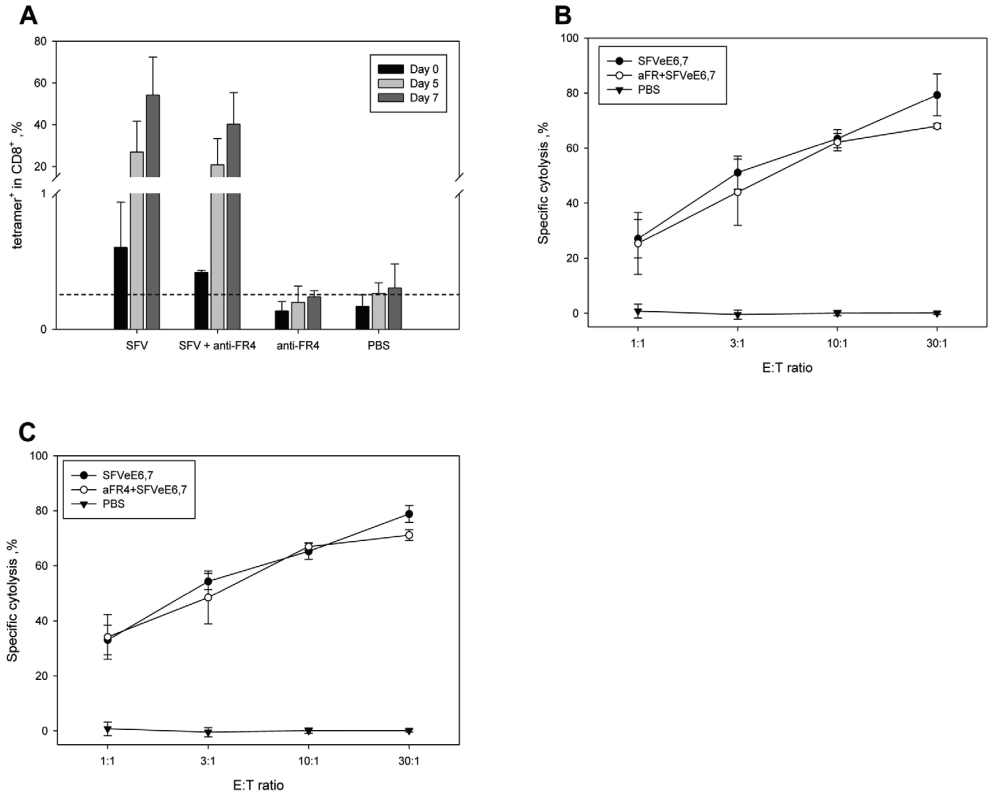


Figure 5. ***In vivo*** **depletion of Treg, with anti-FR4, did not enhance the frequency and the activity of HPV-specific CTL**

Mice were immunized (day 0) and boosted (day 14) i.m. with 10^6 SFVeE6,7 (n=6) or PBS (n=6). In each immunization group three mice also received an i.p. injection with 25 μg anti-FR4 (day -1 and day 13).Ten days after the last injection, the mice were killed and spleen cells were isolated. (A) Freshly isolated splenocytes, as well as 5 and 7 days *in vitro* restimulated cells were analyzed by flow cytometry after staining with RAHYNIVTF-carrying MHC class I tetramers and anti-CD8. The dashed line represents background level. (B) After 5 and (C) 7 days of *in vitro* restimulation, cytolytic activity against C3 target cells was determined in triplicate well assay. The levels of specific cytolysis against the target cells at different E:T ratios are shown. Error bars represent standard deviation.

This result suggests that the *in vivo* priming (i.e. interaction and activation of CD8 T cells by antigen presenting cells) and also subsequent proliferation of HPV-specific $CD8^+$ T cells upon SFVeE6,7 immunization is not or only marginally influenced by $CD4^+Foxp3^+$ suppressor T cells.

We restimulated *in vivo* induced HPV-specific $CD8^+$ T cells to analyze if these T cells, elicited under *in vivo* conditions of decreased Treg levels, differ in their recall proliferation potential and/or cytolytic activity compared to cells obtained in the presence of higher frequencies of $CD4^+Foxp3^+$ Treg. Ten days after the booster immunization the spleens of mice treated with anti-FR4 contained approx. 1.2 % $CD4^+Foxp3^+$ cells compared to approx. 1.7 % $CD4^+Foxp3^+$ in control spleens. To exclude that after the standard 7-day restimulation protocol a possible effect of Treg depletion on the proliferation of cells had faded out, we also

analyzed the cells after a shorter restimulation protocol of 5 days. CTL activity of spleen cells from SFVeE6,7-immunized mice was not affected by *in vivo* depletion of $CD4^+Foxp3^+$ T cells (Figure 5B and C). These results suggest that *in vitro* proliferation of SFVeE6,7-induced T cells is presumably at an optimal level, not being further enhanced by decreased numbers of suppressor cells. However more importantly, these data demonstrate that *in vivo* depletion of Treg does not affect the priming phase of immune responses elicited by SFVeE6,7 immunization.

Therapeutic efficacy of immunization with SFVeE6,7 in combination with Treg depletion

We analyzed if depletion of Treg would stimulate the therapeutic efficacy of SFVeE6,7 *in vivo*. To allow detection of an effect of the elimination of Treg, the immunization protocol was

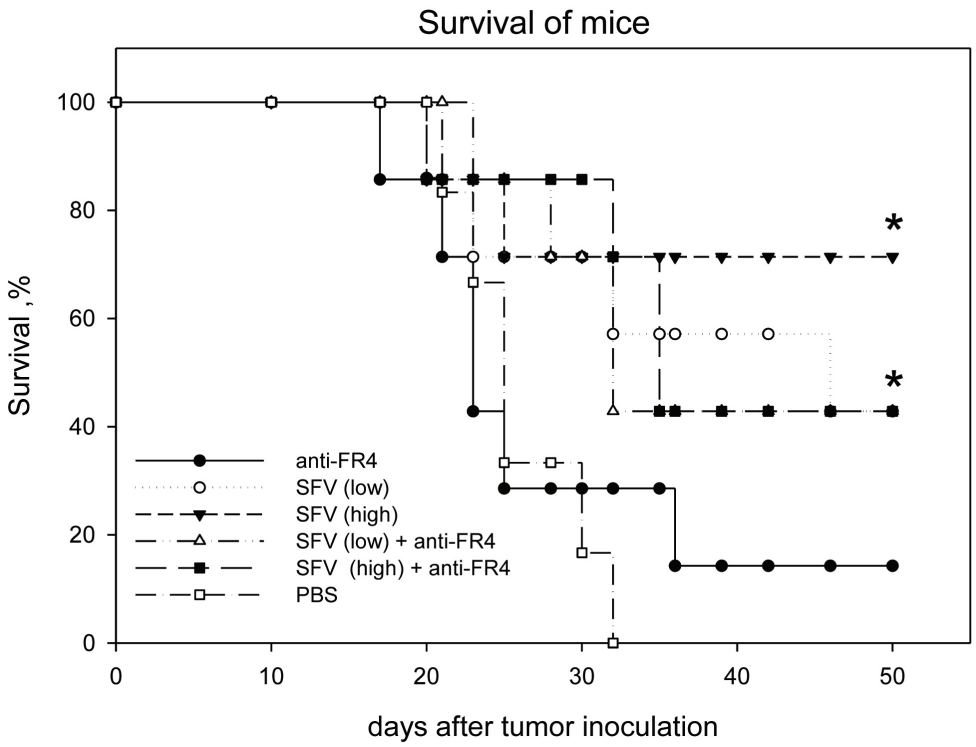


Figure 6. Therapeutic efficacy of SFVeE6,7 immunizations is not enhanced by Treg depletion

Mice were inoculated with $2x10^4$ TC-1 cells and ten days later the mice were i.m. immunized with 10^6 (low dose, n=14) or $5*10^6$ SFVeE6,7 (high dose, n=14) or injected with PBS (n=13). The mice were boosted twice with a one-week interval (day 17 and 24). In each immunization group seven mice also received an i.p. injection with 25 μg anti-FR4, one day before each immunization. Tumor development was monitored twice weekly. When the tumor grew through the skin or when the tumor volume reached a volume of approximately 1000 mm^3, the mice were killed. The effect of the immunization is depicted as the percentage of surviving mice. *$P < 0.05$ as compared with PBS control group.

chosen such that a suboptimal therapeutic effect was attained. We previously have shown that immunization with $5x10^6$ i.u. of SFVeE6,7 at days 7-14 and 21 after tumor inoculation results in the elimination of tumors in 100% of mice.[29] We therefore prime immunized mice 10 days after tumor inoculation with 10^6 i.u. or $5x10^6$ i.u. of SFVeE6,7. The mice were boosted twice, with a one-week interval (day 17 and 24). Part of the mice was also injected with anti-FR4, to deplete Treg, one day before each immunization. Two days after anti-FR4 injection the Treg level within the TC-1 tumor was reduced by 55% compared to control values (data not shown). Starting from day 10, tumor size was measured twice weekly by palpation. All control mice, injected i.m. with PBS, developed tumors and had to be killed within 32 days after tumor inoculation (Figure 6). The group of mice treated with anti-FR4 alone did not show a delay in tumor growth compared to the buffer control mice. All other immunization protocols resulted in delayed tumor growth when compared with the PBS treated group (*$P < 0.05$). No substantial difference in outcome was observed between the groups immunized with SFVeE6,7 alone and groups immunized with SFVeE6,7 combined with Treg depletion. Fifty days after tumor inoculation, 1 and 2 out of 7 mice were tumor-free in groups treated with 10^6 i.u. SFVeE6,7 alone or 10^6 i.u. SFVeE6,7 combined with anti-FR4, respectively. In the groups immunized with the $5x10^6$ of SFVeE6,7 with or without anti-FR4 treatment 2 out of 7 mice did not have tumor 50 days after tumor inoculation. These results demonstrate that depletion of Treg, with anti-FR4, does not improve the therapeutic efficacy of SFVeE6,7 immunization in our murine model system.

Discussion

We focused on the questions of whether SFV-based immunization increases the number and/or activity of Treg and if depletion of Treg enhances efficacy of SFV based immunization strategy in order to assess whether potential clinical application needed concomitant treatment of Treg. Interestingly, SFVeE6,7 immunization did not change numbers or activity of Treg. Furthermore, decreasing Treg numbers *in vitro* and *in vivo*, using the novel, anti-FR4 antibody, did not enhance efficacy of SFVeE6,7 immunization. Both, priming and proliferation of HPV-specific immune cells elicited with the SFVeE6,7 vector is not affected by immunosuppressive activity of Treg. Moreover, Treg depletion did not improve the therapeutic efficacy of SFVeE6,7 in tumor challenge experiments.

Induction of CTL responses using alphavirus vector-based immunization is very efficient.[45-49] In the present study, CTL responses were elicited with as few as 10^5 i.u. of SFV replicon particles, confirming our previous studies.[24,26-29] SFV immunization at dosages that induced potent CTL and antitumor responses did not enhance Treg levels in mice. Notably, even a 100-fold higher dose of SFVeE6,7 (i.e. 10^7 i.u. of SFVeE6,7) did not increase the frequency of Treg in blood and spleen of immunized mice. In contrast, vaccination of mice with recombinant vaccinia virus, dendritic cells, adenylate cyclase, peptide, DNA or Listeria-based vaccines

and humans with dendritic cells resulted in Treg expansion suggesting that these therapeutic cancer vaccines candidates may stimulate tumor-specific T-cell tolerance.[9-14,22,23] The fact that SFV immunization does not expand Treg suggests that this therapeutic cancer vaccine does not induce T-cell tolerance, possibly contributing to its potency.

One can envision several reasons why Treg are not induced upon SFVeE6,7 immunization. Firstly, a relatively low dose of recombinant SFV is required for a therapeutic effect as compared to other recombinant virus vaccines. Prime-boost immunization with as few as $5x10^6$ SFV particles suffices for effective tumor eradication. We previously demonstrated that to induce a therapeutic effect with recombinant adenovirus 100-1000-fold more adenovirus compared to SFV is required.[29] A large amount of viral vector antigen might evoke a strong anti-vector response resulting in some degree of immune suppression. Secondly, upon injection, SFV replicon particles undergo only one round of infection, as RNA encoding structural proteins is not encapsidated in the replicon particles.[48] Thus, as infected cells die through apoptosis within 3 days, expression of the recombinant gene is transient and time of exposure is short. In comparison, Treg induction is more pronounced upon prolonged exposure to low doses of antigen.[9,11] Further studies are required to elucidate which mechanisms or components of vaccines are involved in the induction of Treg.

We demonstrated that *in vitro* proliferation and activity of HPV-specific CTL (induced by SFVeE6,7 immunization) was barely affected by the presence or absence of Treg. Only at a very high, nonphysiological, Treg to spleen cell ratio, suppression of HPV-specific CTL proliferation was observed. On the other hand, Treg strongly decreased the proliferation of non-specific T cells stimulated with anti-CD3 and anti-CD28 indicating that the sorted Treg are functional and, in line with other reports, demonstrates that these cells have the potency to inhibit the proliferation of different subsets of T cells *in vitro*, including $CD8^+$ T cells.[7,40,43,50,51] Thus, while Treg do inhibit non-specific T cell proliferation we observed no marked effect of Treg on the *in vitro* proliferation of SFV-induced HPV-specific CTL.

TH6 antibody (anti-FR4), is a good candidate for selective Treg depletion. This anti-FR4 treatment strongly decreased Treg levels in blood and spleen for a prolonged time. Intraperitoneal injection of (6-8 week-old) mice, with as little as 25 μg of antibody, resulted in 80% reduction of the Treg population in peripheral blood mononuclear cells (PBMCs) within one day. Even after 14 days the population in PBMCs was reduced to 40% of control. Treg depletion in mice using anti-CD25 antibody or low dose cyclophosphamide is not nearly as strong and does not persist for a long time.[7,40,52] For example, Lutsiak *et al.* showed that i.p. cyclophosphamide injection in mice decreased the number of Treg in spleen to 40% of control values on day 4 while already after 10 days Treg level were comparable to those in control mice.[7] Moreover, anti-FR4 injection specifically decreased only $Foxp3^+CD25^+$ cells, leaving the $Foxp3^-CD25^+$ population unchanged. This observation is in line with recent studies and shows that anti-FR4 does not decrease the levels of other activated cells than Treg.[53] Importantly, anti-FR4 administration did not affect $CD8^+$ T cells levels. Studies from our group and

others clearly demonstrate that this population plays a crucial role in tumor immunity.[29,53] It should however be noted that a high dose of anti-FR4 (100 μg) in very young mice (10-20 days of age) induces some degree of autoimmune disease.[43]

It has been demonstrated before, using anti-CD25 antibody, that reduction of the Treg numbers around time point of immunization improves the immune responses induced by the vaccination,[6] however, depletion with anti-CD25 at later time points does not affect immune responses. Therefore we decided to inject anti-FR4 antibody one day before each SFVeE6,7 immunization. The combination of anti-FR4 treatment with SFVeE6,7 immunization, despite the strong reduction in Treg numbers, did not increase number and activity of HPV-specific CTL. We obtained similar results, when depleting Treg with anti-CD25 (PC61 clone) (data not shown). Treg depletion (using anti-FR4) also did not improve the therapeutic efficacy of SFVeE6,7 (in comparison to the group treated with SFV only) in a mouse TC-1 tumor model. By own observation and as reported on by others,[54] TC-1 tumors contain high numbers of intratumoral Treg. Therefore, the lack of improvement in therapeutic efficacy of SFVeE6,7 when combining immunization with Treg depletion can not be explained by the absence of Treg in TC-1 tumors. Tuve *et al.*[54] as well as Chuang *et al.*[55] have also shown that Treg depletion/inhibition alone (using anti-CD25 antibody or cyclophosphamide) is not sufficient to induce efficient antitumor responses against TC-1 tumor cells. By contrast in the latter studies Treg depletion does improve the efficacy of HPV DNA vaccine against TC-1 tumor cells.[55]

In conclusion, while several other immunization strategies benefit from a combination with treatments to reduce Treg, the efficacy of SFVeE6,7 immunization is not enhanced by the depletion of Treg while furthermore the presence of Treg does not seem to affect the SFV-induced immune response. These observations underline the strength of our SFV-based vaccination approach.

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